Nuclear Relocalization of the Pre-mRNA Splicing Factor PSF during Apoptosis Involves Hyperphosphorylation, Masking of Antigenic Epitopes, and Changes in Protein Interactions

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The spatial nuclear organization of regulatory proteins often reflects their functional state. PSF, a factor essential for pre-mRNA splicing, is visualized by the B92 mAb as discrete nuclear foci, which disappeared during apoptosis. Because this mode of cell death entails protein degradation, it was considered that PSF, which like other splicing factors is sensitive to proteolysis, might be degraded. Nonetheless, during the apoptotic process, PSF remained intact and was N-terminally hyperphosphorylated on serine and threonine residues. Retarded gel migration profiles suggested differential phosphorylation of the molecule in mitosis vs. apoptosis and under-phosphorylation during blockage of cells at G1/S. Experiments with the use of recombinant GFP-tagged PSF provided evidence that in the course of apoptosis the antigenic epitopes of PSF are masked and that PSF reorganizes into globular nuclear structures. In apoptotic cells, PSF dissociated from PTB and bound new partners, including the U1–70K and SR proteins and therefore may acquire new functions.

INTRODUCTION

Splicing factors are found within the nucleus both in a diffuse form and in distinct domains termed interchromatin granules (IG) or "speckles" (Spector, 1993). These functional compartments are spatially dynamic with regard to movement and composition (Eils et al., 2000). Splicing factors within IGs are highly mobile and are constantly associating and dissociating from their compartments (Phair and Misteli, 2000). The mAb, B92, which recognizes the polypyrimidine tract binding protein (PTB)-associated splicing factor (PSF), produces typical specked nuclear pattern in immunostaining. These PSF speckles disappear during granulocytic differentiation (Lee et al., 1996; Shav-Tal et al., 2000). We recently showed that this disappearance is associated with partial degradation (Shav-Tal et al., 2000) and by the formation of alternative nuclear structures (Shav-Tal et al., 2001). The present study indicates that PSF speckles disappear also

through a different mechanism, involving conformational changes that cause breakdown of PSF speckles and relocalization of the molecule in the nucleus. The functional nature of speckles is a matter of some controversy (for review, see Park and De Boni, 1999). These structures have been suggested to play a role in storage and recycling of splicing factors (Jimenez-Garcia and Spector, 1993), whereas a portion may act as reservoirs for the recruitment to active sites of transcription (Huang and Spector, 1996; Misteli et al., 1997; Zeng et al., 1997). This view does not predict a direct involvement of speckles in pre-mRNA splicing. On the other hand, it has been shown that nascent mRNA transcripts and transcription sites are closely associated with speckles, that transcription occurs at the surface of speckles (Clemson and Lawrence, 1996), and that microinjected pre-mRNAs have high affinity to speckles (Wang et al., 1991). It has thus been proposed that speckles can act coordinately in transcription and splicing and that pre-mRNA splicing can take place both at the periphery and inside speckles (Smith et al., 1999; Wei et al., 1999). A model combining both views suggests that splicing factors are recruited to transcription sites, whereas certain mRNAs are released from such sites and associate with splicing factor reservoirs (Melcak et al., 2000).

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  Abbreviations used: BM, bone marrow; IG, interchromatin granules; PSF, PTB-associated splicing factor; PTB, polypyrimidine tract binding protein.

The traffic of splicing factors and their relocalization within the nucleus are thus of functional importance. This process appears to be controlled by protein phosphorylation (Misteli and Spector, 1997; Misteli, 1999); serine-arginine (SR) proteins, a major constituent of IGs (Mintz *et al.*, 1999), are phosphoproteins containing a C-terminal serine-arginine rich RS domain and N-terminal RNA recognition motifs (RRMs; for review, see Cáceres and Krainer, 1997). Experiments monitoring GFP-tagged SF2/ASF SR protein in living cells have shown that phosphorylation controls the movement of SR proteins from speckles to sites of active transcription (Misteli *et al.*, 1997, 1998). Similarly, in vitro and in vivo studies using several kinases indicate that phosphorylation of SR proteins releases them from IGs (for review, see Stojdl and Bell, 1999), whereas phosphatases have an opposite effect (Misteli and Spector, 1996). Therefore, it has been proposed (Cáceres *et al.*, 1997; Misteli *et al.*, 1998; Stojdl and Bell, 1999) that the recruitment of splicing factors from speckles occurs in two steps. First, SR proteins are physically released from speckles via RS domain phosphorylation. Subsequently, targeting to sites of transcription occurs via interactions with other proteins and association with pre-mRNA through their RRMs. Disassembly of speckles occurs also during mitosis (Spector *et al.*, 1991), whereas Cajal bodies, nuclear lamins, nuclear pore complexes, and DNA replication sites remain intact (Gui *et al.*, 1994). SR proteins are hyperphosphorylated during metaphase, and this opposes their retention in speckles (Gui *et al.*, 1994).

Although PSF does not posses an RS domain, it shares some features with SR proteins. In the present study we show that hyperphosphorylation of PSF during apoptosis is associated with binding to new partner proteins, thus assigning a possible new function to this splicing factor.

MATERIALS AND METHODS

Bone Marrow Cells and Cell Lines

BM cells were obtained from 6-week-old male or female BALB/c mice (Harlan-Olac, Indianapolis, IN) and dissociated to single-cell suspensions in cold phosphate-buffered saline (PBS). HeLa and HL-60 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and MPC-11 cells in RPMI medium supplemented with 10% FCS.

Protein Extraction, Immunoprecipitation, and Immunoblotting

Cell lines were lysed for 2 h on ice in 50 mM Tris-HCl, pH 8, containing 1% Nonidet P-40 (NP-40), 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methylsulfonyl fluoride (PMSF), 3 μg/ml aprotinin, 20 μg/ml leupeptin, 10 mM iodoactetate, 1 mM sodium orthovanadate, and 10 mM sodium fluoride. BM cells were lysed by boiling immediately after addition of SDS-sample buffer (5% glycerol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 2% 2-mercaptoethanol, 0.01% bromophenol blue). For immunoprecipitation, cell lysates were clarified by centrifugation at 15,000 × *g* for 10 min at 4°C. The resulting supernatants were precleared with protein A-Sepharose (Sigma, St. Louis, MO) and were immunoprecipitated at 4°C with the indicated antibody and protein A-Sepharose. Immunoprecipitates were washed (4 times) with cold SNNTE buffer (5% sucrose, 1% NP-40, 500 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA), boiled in SDS sample buffer, and analyzed by SDS-PAGE. Immunoblot analysis of tissue and cell lysates (50 μg/lane) was performed as described (Peled *et al.*, 1991). Specific binding was detected with horseradish peroxidase (HRP)-coupled antibodies and enhanced chemiluminescence (ECL) reagents.

Cell-cycle Blockage and Apoptosis

MPC-11 cells were thymidine (G1/S)-blocked (5 mM, Sigma) for 24 h. The cells were then washed and incubated in medium without thymidine for 2 h before blocking at metaphase by 50 ng/ml nocodazole for 14 h. HeLa cells were thymidine-blocked (2 mM, 8 h) followed by nocodazole block (50 ng/ml, 14 h). Mitotic cells were obtained by mechanical shake-off, and purity (>90%) was checked by DNA staining. HL-60 cells were induced to undergo apoptosis with the use of 5 μg/ml actinomycin D for 4 h. Apoptotic HL-60 cells were separated from nonapoptotic cells by a Percoll density gradient as previously described (Martin *et al.*, 1990). Presence of apoptotic cells (>50%) after 4 h of treatment was confirmed by DNA gel electrophoresis as in Park and Patek (1998), by staining with Hoechst nuclear staining for detection of nuclear condensation and fragmentation and by staining with Annexin-V-Alexa 568 (Boehringer Mannheim, Mannheim, Germany), which detects phosphatidylserine on the membranes of apoptotic cells

2-D Phosphoamino Acid Analysis and Phosphopeptide Mapping

Metabolic labeling with ^{32}P-orthophosphate (250 μCi/ml; Amersham, Arlington Heights, IL) was performed in phosphate-free DMEM with untreated cells, mitotically arrested HeLa cells, and apoptotic HL-60 cells. PSF was immunoprecipitated from these cells, run on SDS-PAGE, and transferred to a PVDF membrane. Excision of the bands, 2-D phosphoamino acid analysis, and phosphopeptide mapping were performed as described (Van Der Geer and Hunter, 1994). For phosphopeptide mapping, phosphorylated PSF was digested by chymotrypsin and peptides were oxidized. Peptides were run in buffer, pH 4.72, and then in phosphochromatography buffer (Van Der Geer and Hunter, 1994). For phosphatase treatment, 40 μg of protein extract were incubated with 400 U of λ protein phosphatase (New England Biolabs, Beverly, MA) at 30°C for 1 h.

Antibodies

Secondary antibody used for Western blotting was goat anti-mouse HRP (Sigma). Fluorophore-labeled secondary antibody used was donkey anti-mouse FITC (Jackson, West Grove, PA). Anti-GFP mAb (JL-8) was purchased from Clontech (Palo Alto, CA). Anti-U1–70K antibody and anti-SR proteins antibody (Mab 104) were provided by Dr. Gil Ast (Tel Aviv University). Anti-PTB mAb was provided by Dr. David Helfman (Cold Spring Harbor). B92 mAb was prepared as previously described (Lee *et al.*, 1996). Polyclonal anti-PSF 1121 antibody was prepared as previously described (Shav-Tal *et al.*, 2000).

Immunofluorescence

Cells were fixed for 2 min in 4% paraformaldehyde with 0.5% Triton X-100 and for an additional 20 min in 4% paraformaldehyde. After washing and blocking in 5% BSA, cells were stained with the indicated antibody for 45 min and then with the appropriate fluorophore antibody for 45 min and counterstained with Hoechst. Immunofluorescence was determined in a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany).

Mouse PSF Cloning

A mouse fibroblast ZAP-Express library was divided in 20 pools and screened by PCR with the use of PSF-specific primers and vector-specific primers. Plaque lifts were prepared from positive pools containing large PSF clones and probed on filters (Schleicher

& Schuell, Dassel, Germany). One large 1.7-kb clone containing the middle to C-terminal part of mouse PSF was isolated and sequenced. The N-terminal GC-rich portion of PSF was cloned with the use of RT-PCR with 3′ primers from published mouse PSF sequences and 5′ primers from mouse PSF-related sequences found in the EST database (mouse PSF sequence-GenBank accession number AY034062).

Transfections

HeLa cells were plated at 70% confluency in six-well plates and transfected with 6 μg of human GFP-PSF constructs with the use of the calcium phosphate transfection method (Graham and Eb, 1973). Cells were fixed and processed for immunofluorescence 24 h after transfection with the use of a Zeiss microscope and photographed by a CCD camera.

RESULTS

Nuclear Localization of Mouse and Human PSF: Reduced Detection by an mAb During Mitosis and Apoptosis

It is well documented that the speckled pattern, as visualized by use of antibodies to several splicing factors, is lost in dividing cells. Splicing factors remain intact and are diffusely dispersed throughout the cytoplasm but also concentrate in a few cytoplasmic foci (Spector *et al.*, 1991 and references within). Speckles reassemble toward the end of mitosis. We examined the nuclear distribution of the essential splicing factor PSF during mitosis of human and mouse cells. During interphase PSF localized in nuclear speckles in immature human HL-60 myeloid leukemia cells (Figure 1A). PSF foci scattered throughout the cytoplasm in late prophase (Figure 1B), which is an intermediate stage during which transcription ceases (Gottesfeld and Forbes, 1997), the chromatin condenses, and the nuclear envelope breaks down (Newport and Spann, 1987). As mitosis proceeded, the PSF immunostaining diminished (Figure 1C) and completely disappeared during metaphase and anaphase (Figure 1, D and E). Western blot analysis clearly demonstrated that this disappearance was not due to protein degradation (Figure 2,A and B). Thus, although PSF remains intact in mitosis, it becomes inaccessible to the B92 antibody. In mitotic cells from mouse bone marrow, PSF-speckled staining diminished, yet in contrast to the findings with the human HL-60 cells, some staining was concentrated at the rim of the metaphase chromosomes (Figure 1, G and H). During anaphase PSF staining reappeared and speckles began to reassemble (Figure 1I). Differences between human and mouse in immunostaining with antibodies to PSF have been documented in our previous studies on maturing granulocytes from mouse bone marrow. The common speckled distribution observed in immature granulocytes disappears in mature and segmented granulocytes (Lee *et al.*, 1996), although the protein remains intact (Shav-Tal *et al.*, 2001). On the other hand, in human peripheral blood granulocytes immunostaining with anti-PSF antibodies produces a clear specked pattern (Shav-Tal and Zipori, unpublished results). These data suggested possible structural differences between the human and mouse proteins. The middle region of mouse PSF has previously been cloned (Chanas-Sacré *et al.*, 1999; Shav-Tal *et al.*, 2000), and its amino acid sequence is 98% identical to human PSF (Patton *et al.*, 1993). However, the GC-rich N-terminus and the C-terminus remained unidentified. We obtained the entire sequence of mouse PSF mRNA by cloning from a mouse fibroblast library (Figure 3A). Mouse and human PSF cDNAs were found to be highly homologous (Figure 3B), and in total, mouse PSF protein is 8 amino acids shorter than human PSF. The C-terminal region containing the functional domains is almost identical in mouse and human, whereas in the N-terminus several differences are observed, although the structural elements (P and Q stretches) are conserved (Figure 3B). It has been suggested (Patton *et al.*, 1993) that the N-terminal region of PSF, which has high proline and glutamine content, has the characteristics of domains involved in protein–protein interactions (Courey and Tjian, 1988; Tanese *et al.*, 1991). Thus, the differences in sequences may lead to different interactions and to differences in localization.

Lack of detection of PSF by the B92 antibody, such as that observed in mouse metaphase cells, was also evident and was a more striking occurrence in HL-60 cells undergoing spontaneous apoptosis (Figure 4, A and B). We considered that this could be due to complete degradation of the protein in apoptosis because of the high sensitivity of PSF to proteases. However, Western blotting indicated that PSF remains intact in apoptotic cells, whereas PTB is cleaved (Shav-Tal *et al.*, 2000). Thus, both in apoptotic cells and in mitotic cells PSF speckles disappear; however, no detectable diffuse staining is observed, as previously described for other splicing factors (Spector *et al.*, 1991). These data imply a possible change occurring in PSF that modifies the antigenic epitope recognized by the B92 antibody.

Overexpressed GFP-tagged PSF Localizes in Apoptotic Bodies Inaccessible to Anti-PSF Antibodies

Overexpression of tagged recombinant PSF could provide a tool to identify the protein, regardless of any conformational changes it may undergo during the cell cycle or in apoptosis. We therefore used human GFP-PSF (Dye and Patton, 2001) that localized in speckles in interphase nuclei and also produced a diffuse nucleoplasmic staining (Figure 4, C and D). In mitotic cells part of the GFP-PSF protein was concentrated in several rounded structures, whereas some was diffusely distributed throughout the cytoplasm (Figure 4, E and F). In apoptotic cells, identified by Annexin-V staining, GFP-PSF concentrated in several large round aggregates in each cell, and no diffuse staining was observed outside of these structures (Figure 4, G–I). In addition, we found in G1/S thymidine-blocked cells that cannot enter into mitosis, that GFP-PSF localized in stellate-like structures rather than in speckles (Figure 4J). To assess the availability of GFP-PSF structures to the B92 antibody, G1/S-blocked and apoptotic cells expressing GFP-PSF, were stained with this anti-PSF antibody (B92). In G1/S-blocked cells the B92 antibody stained the GFP-PSF structures and showed an image identical to that produced by GFP (Figure 4, J–L). Thus, in G1/S-blocked cells PSF retained the antigenic epitope recognized by the B92 antibody. On the other hand, in apoptotic cells, B92 did not stain the large GFP-PSF structures (Figure 4, M–O). It is thus evident that during apoptosis PSF is modified to the extent that it is totally unrecognized by the B92 antibody. This is not occurring in mitotic cells in which antigenicity is preserved.
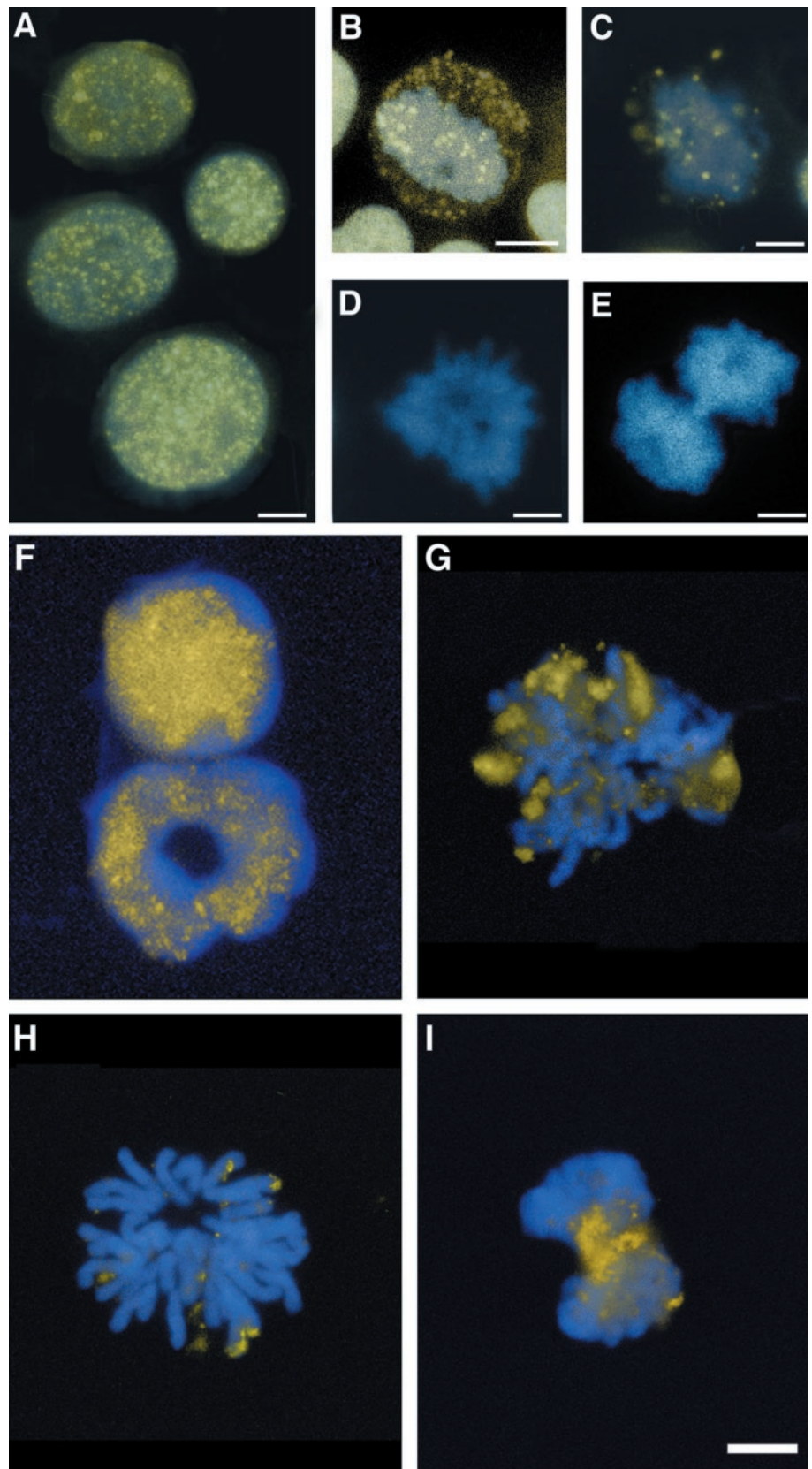
Figure 1. PSF localization during cell cycle in human leukemic cells and mouse hemopoietic cell. HL-60 cells were stained for immunofluorescence with the B92 antibody. (A) Interphase nuclei with PSF staining (B92 in yellow) on the background of Hoechst DNA stain (blue). (B) A nucleus at late prophase showing chromatin condensation, in which nuclear membrane breakdown has occurred and PSF speckles are found in the cytoplasm. (C) Nucleus in prometaphase; (D) metaphase; (E) anaphase. Bone marrow cells were stained for immunofluorescence with the B92 antibody. (F) Interphase nuclei of an immature cell and a mature granulocyte exhibit a speckled pattern. (G) Prometaphase; (H) metaphase; (I) anaphase. Bar, 5 μm. (Artificial coloration was achieved by computer coloring of overlapping black and white pictures of FITC staining and Hoechst staining taken from the same field).
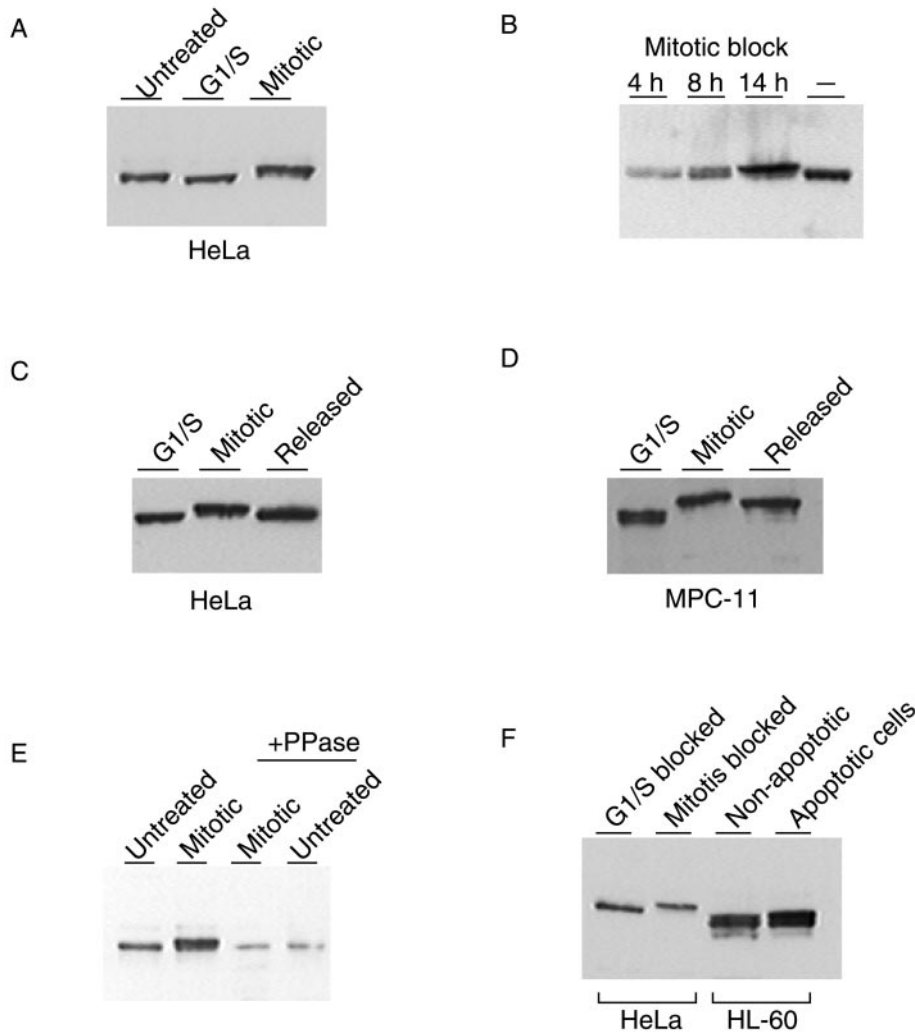
Figure 2. Phosphorylation of PSF during mitosis and apoptosis. HeLa cells were blocked at G1/S by a thymidine block followed by a nocodazole block causing cells to stop at metaphase. (A) Protein extracts were made from untreated and treated cells, and blots were probed with the B92 antibody. (B) Extracts were made from HeLa cells treated with nocodazole for the indicated time periods. (C) The cells were then released from the mitotic block, and extracts were made after a few hours. (D) Mouse MPC-11 cells underwent the same treatments. (E) Mitotically blocked HeLa protein extracts were treated with λ protein phosphatase at 30°C for 1 h. (F) HL-60 apoptotic cells were separated from nonapoptotic cells with the use of a Percoll gradient. The shift in the apoptotic cells was compared with mitotic cells. All the shifts observed were reproducible.

It has been shown that phosphorylated SR proteins are associated with the U1-snRNP complex during apoptosis (Utz *et al.*, 1998). We thus decided to check whether endogenous PSF might bind to different partners during apoptosis. Part of nuclear PSF is normally found in complex with the PTB protein (Patton *et al.*, 1993). PTB is proteolytically cleaved during apoptosis (Shav-Tal *et al.*, 2000) and indeed is not found in complex with PSF in these cells (Figure 5A). On the other hand, immunoprecipitation experiments showed that PSF is associated with the U1–70K protein and SR proteins in apoptotic cells (Figure 5, B–D). Thus, the masking of antigenic sites during apoptosis may be due to interactions with new proteins.

PSF Is Hyperphosphorylated during Apoptosis

One possible modification of PSF that may account for acquisition of new protein binding capabilities could be its degree of phosphorylation. Treatment of HL-60 cells with actinomycin D for >4 h leads to apoptosis (Martin *et al.*, 1990). Apoptotic cells were identified and separated from nonapoptotic cells as described in MATERIALS AND METHODS (data not shown). We found that PSF from apoptotic protein cell extracts, presented retarded migration of PSF compared with cells possessing a normal nucleus (Figure 2F). Human HeLa and mouse plasmacytoma (MPC-11) cells were synchronized to G1/S and then blocked at M phase. Retarded migration of the PSF band was seen in these states. The 100-kDa PSF band from nocodazole-blocked HeLa cells (metaphase) migrated more slowly than that from untreated cells, and both of the above ran slower than PSF from thymidine-blocked cells (interphase; Figure 2A). The shift in mitotic cells was best observed after a 14-h block (Figure 2B). When the mitotic block was released, the PSF band was shifted back (Figure 2C). The same features were seen in the mouse MPC-11 cells (Figure 2D). Treatment of the mitotic protein extracts with Lambda phosphatase showed a faster migrating PSF band (Figure 2E), thus indicating that the change in PSF migration is probably due to phosphorylation and that PSF can be found in different phosphorylation states. Although we observed hyperphos-

A
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1    CTTCTGCCTCGACCGCCCCTTGACTACAGCTATGTCTCGGGATCGGTTCCGGAGTCGCGG 60
1                                  M  S  R  D  R  F  R  S  R  G  10

61   TGGCGGCGGTGGAGGCTTTCACCGGCGTGGAGGAGGTGGCGGCCGCGGCGGCCTTCACGA 120
11   G  G  G  G  G  F  H  R  R  G  G  G  G  G  G  R  G  G  L  H  D  30

121  CTTCCGCTCTCCGCCGCCGGGGCATGGGCCTCAACCAGAACCGCGGCCCCATGGGCCCGGG 180
31   F  R  S  P  P  P  G  M  G  L  N  Q  N  R  G  P  M  G  P  G  50

181  CCCTGGCGGCCCGAAGCCGCCGCTCCCGCCTCCACCTCCTCACCAGCAGCAGCAGCCA 240
51   P  G  G  P  K  P  P  L  P  P  P  P  H  Q  Q  Q  Q  P  70

241  GCCGCCGCAGCAGCCTCCGCCGCAGCAGCCGCCGCCGCACCAGCAGCCGCCGCCGCACCA 300
71   P  P  Q  Q  P  P  P  Q  Q  P  P  P  H  Q  Q  P  P  P  H  Q  90

301  GCCGCCCCATCAACAGCCCCCGCCTCCGCCGCAGGAATCCAAGCCCGTCGTCCCCCAAGG 360
91   P  P  H  Q  Q  P  P  P  P  Q  E  S  K  P  V  V  P  Q  G  110

361  CCCCGGCTCGGCGCCGGGGGTGAGCAGTGCGGCCTCCGCCGGCGGTCTCGGCTCCGCCCGC 420
111  P  G  S  A  P  G  V  S  S  A  P  P  P  A  V  S  A  P  P  A  130

421  CAACCCCCCGACCACCGGCGCCCCTCCGGGGCCCTGGTCCAACCCCGACTCCGCCGCCCGC 480
131  N  P  P  T  T  G  A  P  P  G  P  G  P  T  P  T  P  P  P  A  150

481  CGTCCCCTCCACCGCCCCCGGACCGGCCTCCCCCATCGACGCCGAGCAGCGGAGTCTCGAC 540
151  V  P  S  T  A  P  G  P  P  P  P  S  T  P  S  S  G  V  S  T  170

541  CACCCCTCCACAGACCGGCGGCCCTCCGCCACCGCCCGCCGGGGGCGCCGGGCCGGGGCC 600
171  T  P  P  Q  T  G  G  P  P  P  P  A  G  G  A  G  P  G  P  190

601  TAAGCCGGGGCCAGGCCCTGGCGGTCCAAAAGGCGGCAAGATGCCCGGTGGGCCTAAGCC 660
191  K  P  G  P  G  P  G  G  P  K  G  G  K  M  P  G  G  P  K  P  210

661  TGGAGGTGGCCCGGGCATGGGCGCTCCTGGTGGCCACCCGAAGCCACCACACCGAGGTGG 720
211  G  G  G  P  G  M  G  A  P  G  G  H  P  K  P  P  H  R  G  G  230

721  CGGCGAGCCCCGTGGGGGCCGGCAGCATCATGCGCCCTACCACCAGCAGCACCACCAGGG 780
231  G  E  P  R  G  G  R  Q  H  H  A  P  Y  H  Q  Q  H  Q  G  250

781  GCCCCCTCCCGGCGGACCGGGACCGGCACGGAGGAGAAGAATCTTCCGACTCGGAGGGGATT 840
251  P  P  P  G  G  P  G  P  R  T  E  E  K  I  S  D  S  E  G  F  270

841  TAAAGCCAACTTGTCTCTCTTGCGGAGGCCTGGAGAAAAAACTTACACACAGCGCTGTCG 900
271  K  A  N  L  S  L  L  R  R  P  G  E  K  T  Y  T  Q  R  C  R  290

901  GTTGTTTGTGGGGAATCTACCTGCTGATATCACAGAGGATGAATTCAAAAGACTGTTTGC 960
291  L  F  V  G  N  L  P  A  D  I  T  E  D  E  F  K  R  L  F  A  310

961  TAAATACGGAGAACCCGGAGAAGTTTTTATCAACAAAGGCAAAGGGTTCGGGTTCATTAA 1020
311  K  Y  G  E  P  G  E  V  F  I  N  K  G  K  G  F  G  F  I  K  330

1021 GCTTGAATCTAGAGCCCTTGGCTGAAATCGCCAAAGCTGAGCTTGATGATACTCCCATGAG 1080
331  L  E  S  R  A  L  A  E  I  A  K  A  E  L  D  D  T  P  M  R  350

1081 AGGTAGACAGCTTCGGGTTCGATTTGCCACACACGCTGCAGCCCTGTCTGTTCGAAATCT 1140
351  G  R  Q  L  R  V  R  F  A  T  H  A  A  A  L  S  V  R  N  L  370

1141 CTCTCCTTATGTTTCCAACGAACTTTTGGAAGAGGCCTTTAGCCAGTTTGGTCCTATTGA 1200
371  S  P  Y  V  S  N  E  L  L  E  E  A  F  S  Q  F  G  P  I  E  390
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1201 AAGGGCTGTTGTAATTGTGGATGATCGCGGAAGATCTACAGGGAAAGGCATTGTTGAGTT 1260
391  R  A  V  V  I  V  D  D  R  G  R  S  T  G  K  G  I  V  E  F  410

1261 TGCTTCCAAGCCAGCAGCAAGAAAAGCATTTGAAAGATGCAGTGAAGGTGTTTTCCTACT 1320
411  A  S  K  P  A  A  R  K  A  F  E  R  C  S  E  G  V  F  L  L  430

1321 GACAACGACTCCTCGCCCAGTCATTGTTGAACCACTTGAACAGTTAGATGACGAAGATGG 1380
431  T  T  T  P  R  P  V  I  V  E  P  L  E  Q  L  D  D  E  D  G  450

1381 TCTTCCTGAAAAGCTGGCCCAGAAGAATCCAATGTATCAAAAGGAGAGAGAAACACCACC 1440
451  L  P  E  K  L  A  Q  K  N  P  M  Y  Q  K  E  R  E  T  P  P  470

1441 TCGTTTTGCTCAGCATGGCACATTTGAGTATGAATATTCTCAACGATGGAAGTCCTTGGA 1500
471  R  F  A  Q  H  G  T  F  E  Y  E  Y  S  Q  R  W  K  S  L  D  490

1501 TGAAATGGAAAAACAGCAAAGGGAACAAGTTGAAAAAAACATGAAGGATGCTAAGACAA 1560
491  E  M  E  K  Q  Q  R  E  Q  V  E  K  N  M  K  D  A  K  D  K  510

1561 ATTGGAAAGTGAAATGGAAGATGCTTACCATGAACACCAAGCAAATCTTTTGCGCCAAGA 1620
511  L  E  S  E  M  E  D  A  Y  H  E  H  Q  A  N  L  L  R  Q  D  530

1621 TCTGATGAGACGCCAGGAAGAATTAAGGCGCATGGAGGAACTTCACAGTCAAGAAATGCA 1680
531  L  M  R  R  Q  E  E  L  R  R  M  E  E  L  H  S  Q  E  M  Q  550

1681 GAAACGTAAAGAAATGCAGTTGAGGCAAGAGGAGAGAAGGCGTAGACGAGGAAGAGAGAT 1740
551  K  R  K  E  M  Q  L  R  Q  E  E  R  R  R  R  E  E  E  M  570

1741 GATGATTCGCCAACGTGAGATGGAAGAACAAATGAGACGCCAACGAGAAGAAAGTTACAG 1800
571  M  I  R  Q  R  E  M  E  E  Q  M  R  R  Q  R  E  E  S  Y  S  590

1801 CAGGATGGGCTACATGGATCCAAGAGAAAGAGACATGAGAATGGGTGGTGGTGGAACAAT 1860
591  R  M  G  Y  M  D  P  R  E  R  D  M  R  M  G  G  G  G  T  M  610

1861 GAACATGGGAGATCCCTATGGTTCAGGAGGCCAGAAATTTCCACCACTAGGTGGTGGTGG 1920
611  N  M  G  D  P  Y  G  S  G  G  Q  K  F  P  P  L  G  G  G  G  630

1921 TGGCATAGGTTATGAAGCTAATCCTGGAGTTCCACCAGCAACCATGAGTGGTTCCATGAT 1980
631  G  I  G  Y  E  A  N  P  G  V  P  P  A  T  M  S  G  S  M  M  650

1981 GGGGAAGCGACATGgGTACTGAGCGCTTTGGGCAGGGAGGTGCGGGTCCTGTGGGTGGACA 2040
651  G  S  D  M  R  T  E  R  F  G  Q  G  G  A  G  P  V  G  G  Q  670

2041 GGGTCCTAGAGGGAATGGGGCCTGGAACTCCAGCAGGATATGGTAgAGGGAGGAGAAGAGTA 2100
671  G  P  R  G  M  G  P  G  T  P  A  G  Y  G  R  G  R  E  E  Y  690

2101 TGAAGGGCCAAATAAAAAACCCCGATTTTAGATATTTAGGCTTTCATTCTAGTTTGGTTT 2160
691  E  G  P  N  K  K  P  R  F  *                                 700

2161 TGTCTCTTTTCTTGTTTAGATACCACTTAAATTCTTGGCATTTTAGTAAGCAAGCTACCTTT 2220

2221 TTATGGATGTTAGCAGTTTATTGACCTGATATTTGTAAATGGCCTGTTTGGGCAGGTAAA 2280

2281 ATTATGTAACGCAGTGTTCAAAACAGGAGAAAAAATTTTTTCATTTACTTTTTTTTTTTAA 2340

2341 CTGTATAATGTTTCTCAAGTTAATGGCAGTGTACCTTGTGCCACCGAATTTCCAAAGTGT 2400

2401 ACCATTTTTTTTTTTACTGTGCTTCAAATAAATAGAAAAAATAGTTATAAAAAAAAAAAA 2460

2461 AAAA 2464
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B
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mPSF 1  MSRDRFRSRGGGGGGFHRRGGGGGRGGLHDFRSPPPGMGLNQNRGPMGPG 50
        ||||||||||||||||||||||||||||||||||||||||||||||||||
hPSF 1  MSRDRFRSRGGGGGGFHRRGGGGGRGGLHDFRSPPPGMGLNQNRGPMGPG 50

51   PG..GPKPPLPPPPHQQQQPPPQQPPPQQPPHQQPPPHQQPPH..QQP 96
     ||  ||||||||||||||||||||||||||||||||  |||| ||  ||
51   PGQSGPKPPIPPPPHQQQQPPPQQPPPQQPPPH.QPPPHQPQHQQQQP 99

97   PPPPQE.SKPVVPQGPGSAPGVSSAPPPAVSAPPANPPTTGAPP..GPGP 143
     |||| |  |||||||  || ||||||| ||   || ||  ||  ||
100  PPPPQDSSKPVVAQGPGPAPGVGSAPASSSAPPATPPTSGAPPGSGPGP 149

144  TPTPPPAVPSTAPGPPPPSTPSSGVSTTPPQTGGPPPPPAG..GAGPPK 191
     |||||||| |   ||||||||||||| ||  |||  ||||    ||||
150  TPTPPPAVTSAPPGAPPPTPPSSGVPTTPPQAGGPPPPPAAVPGPGPGPK 199

192  PGPGPGGPKGGKMPGGPKPGGGPGMGAPGGHPKPPHRGGGEPRGGRQHHA 241
     ||||||||||||||||| ||||  ||||||||||||||||||||||||
200  QGPGPGGPKGGKMPGGPKPGGGPGLSTPGGHPKPPHRGGGEPRGGRQHHP 249

242  PYHQQHHQGPPPGGPGPRTEEKISDSEGFKANLSLLRRPGEKTYTQRCRL 291
     ||||||| |||||||||| |||||||||||||||||||||||||||||
250  PYHQQHHQGPPPGGPGGRSEEKISDSEGFKANLSLLRRPGEKTYTQRCRL 299

292  FVGNLPADITEDEFKRLFAKYGEPGEVFINKGKGFGFIKLESRALAEIAK 341
     ||||||||||||||||||||||||||||||||||||||||||||||||
300  FVGNLPADITEDEFKRLFAKYGEPGEVFINKGKGFGFIKLESRALAEIAK 349
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342  AELDDTPMRGRQLRVRFATHAAALSVRNLSPYVSNELLEEAFSQFGPIER 391
     ||||||||||||||||||||||||||||||||||||||||||||||||
350  AELDDTPMRGRQLRVRFATHAAALSVRNLSPYVSNELLEEAFSQFGPIER 399

392  AVVIVDDRGRSTGKGIVEFASKPAARKAFERCSEGVFLLTTTPRPVIVEP 441
     ||||||||||||||||||||||||||||||||||||||||||||||||
400  AVVIVDDRGRSTGKGIVEFASKPAARKAFERCSEGVFLLTTTPRPVIVEP 449

442  LEQLDDEDGLPEKLAQKNPMYQKERETPPRFAQHGTFEYEYSQRWKSLDE 491
     ||||||||||||||||||||||||||||||||||||||||||||||||
450  LEQLDDEDGLPEKLAQKNPMYQKERETPPRFAQHGTFEYEYSQRWKSLDE 499

492  MEKQQREVEKNMKDAKDKLESEMEDAYHEHQANLLRQDLMRRQEELRRM 541
     ||||||||||||||||||||||||||||||||||||||||||||||||
500  MEKQQREVEKNMKDAKDKLESEMEDAYHEHQANLLRQDLMRRQEELRRM 549

542  EELHSQEMQKRKEMQLRQEEERRRREEEMMIRQREMEEQMRRQREESYSR 591
     ||||  ||||||||||||||||||||||||||||||||||||||||||
550  EELHNQEMQKRKEMQLRQEEERRRREEEMMIRQREMEEQMRRQREESYSR 599

592  MGYMDPRERDMRMGGGGTMNMGDPYGSGGQKFPPLGGGGIGYEANPGVP 641
     ||||||||||||||||||   |||||||||||||||||||||||||||
600  MGYMDPRERDMRMGGGGAMNMGDPYGSGGQKFPPLGGGGIGYEANPGVP 649

642  PATMSGSMMGSDMRTERFGQGGAGPVGGQPRGMGPGTPAGYGRGREEYE 691
     ||||||||||||||||||||||||||||||||||||||||||||||||
650  PATMSGSMMGSDMRTERFGQGGAGPVGGQPRGMGPGTPAGYGRGREEYE 699

692  GPNKKPRF 699
     ||||||||
700  GPNKKPRF 707
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Figure 3. Cloning of mouse PSF cDNA. (A) The cDNA sequence of mouse PSF was obtained with the use of library screening and RT-PCR. (B) Comparison of mouse and human amino acid sequences.

phorylation in both apoptosis and mitosis, a comparison of the shifted bands implies different levels or sites of PSF phosphorylation in these two different cellular states.

Phosphoamino acid analysis of untreated cells vs. apoptotic or mitotic cells showed that in all cases the majority of phosphorylation on PSF was found on serine residues and to

Figure 4. PSF nuclear localization during apoptosis and mitosis. (A) Untreated HL-60 cells stained with the B92 antibody. (B) An apoptotic nucleus is seen by Hoechst DNA counterstaining. HeLa cells were transfected with GFP-PSF (C); DNA counterstain (D). Transfected cells were synchronized to metaphase by a nocodazole block (E); DNA counterstain (F). Apoptotic cells were identified by membranal Annexin V staining (G) and GFP-PSF was seen to localize in large aggregates (H); DNA counterstain (I). GFP-PSF was transfected into cells that were then blocked at G1/S (J) and double stained with the B92 antibody (K); DNA counterstain (L). Apoptotic cells from a culture transfected with GFP-PSF (M) were double-stained with B92 (N), DNA (O). Bar, 5 μm.
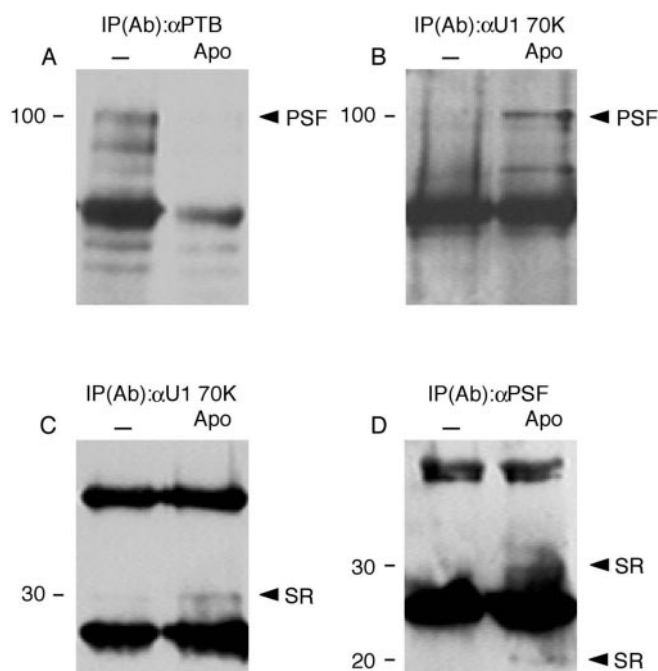
Figure 5. Analysis of binding partners of PSF. HL-60 cells were incubated with actinomycin D for 5 h when more than 50% of the cells become apoptotic. Lysates from apoptotic (Apo) and nonapoptotic (−) cells were immunoprecipitated with an anti-PTB antibody and Western blotted with the B92 antibody (A), immunoprecipitaed with anti–U1–70K and blotted with B92 (B), immunoprecipitated with anti–U1–70K and blotted with anti-SR (C), or immunoprecipitated with the B92 antibody and Western blotted with anti-SR antibodies (D).

a lesser extent on threonine residues (Figure 6A). Phosphoamino acid analysis of the 68-kDa degradation product of PSF found in human cells showed high reduction in threonine phosphorylation, thus mapping the sites of threonine phosphorylation to the degradable part of PSF, i.e., the C terminus (Shav-Tal *et al.*, 2000).

Because no major differences were observed between the different forms of PSF in phosphoamino acid analysis, phosphorylated PSF was subjected to phosphopeptide mapping. This was performed in HL-60 apoptotic cells only, because PSF in HeLa cells did not incorporate ^{32}P efficiently. As can be seen by comparing Figure 6B to 6C, two additional peptides (arrows) appeared in apoptotic cells. These are hydrophobic and large peptides because they migrate only in the organic phase and are probably derived from the same peptide that is additionally phosphorylated in apoptotic cells, thus multiply phosphorylated in a cluster of sites. Because the chymotrypsin used to produce the peptides cannot digest the N-terminal, proline-rich core of PSF, we predict that the large, nonmigrating peptide (No. 7 in Figure 6B) is the N-terminus of PSF. The additional phosphorylated peptides (Nos. 5 and 6 in Figure 6C) resulting from this peptide may therefore be the cause of the changes leading to the lack of detection by our antibody.

To verify that the hyperphosphorylation of PSF takes place in the N-terminus, different GFP-PSF vectors were used in transfections. Figure 7C shows that both full-length

GFP-PSF (amino acids 1–707) and endogenous PSF are detected by the B92 antibody in Western blots, and retarded migration of both proteins is observed in nocodazole-arrested cells (metaphase). Because phosphorylation is thought to control the localization of splicing factors in speckles, we checked whether the removal of RRM2 of PSF, which is responsible for PSF targeting to speckles (Dye and Patton, 2001; Figure 7, D and F), is the region hyperphosphorylated during mitosis. However, although GFP-PSF ΔRRM2 does not localize in speckles, it was also hyperphosphorylated during metaphase (Figure 7F). We then proceeded to check the behavior of the C-terminal half of PSF during mitosis. GFP-PSF 338–707, which can localize in the nucleus (Figure 7, G and H), did not show any shift in mitotic cells (Figure 7I), indicating that hyperphosphorylation occurs on the N-terminal half of PSF. In this case anti-GFP antibody was used for Western blotting because our antibody recognizes only the N-terminus of PSF.

DISCUSSION

Apoptosis entails proteolysis of several regulatory molecules and subsequent protein degradation as part of the process that lends to cessation of cellular functions and cell death (Stroh and Schulze-Osthoff, 1998). Splicing factors are particularly sensitive to proteolytic degradation (La Branche *et al.*, 1991; Casciola-Rosen *et al.*, 1994; Waterhouse *et al.*, 1996; Shav-Tal *et al.*, 2000). In this context it is intriguing that PSF remains intact at a time point in the apoptotic process in which PTB, which is a binding partner to PSF, is cleaved (Shav-Tal *et al.*, 2000). We show in the present study that the B92 mAb failed to immunostain apoptotic cells despite the fact that the relative amount of the PSF protein in the cells remained unchanged. One possible explanation of this phenomenon was that the protein is diffusely distributed within the cell and the local concentration is therefore below the detection power of the immunofluorescence method used. This seems to be the case with mitotic cells. Most of the endogenous PSF protein becomes undetectable with the exception of distinct structures, whereas overexpression of GFP-PSF allowed to visualize the protein both in a diffuse form and in a few round bodies at the margin of chromosomes. Similarly, GFP-PSF–expressing cells blocked at G1/S exhibit unique PSF stellate structures, as well as diffuse green fluorescent staining, both of which are exactly duplicated by staining with the B92 antibody. By sharp contrast, in apoptotic cells, GFP-PSF aggregates in large rounded structures with no diffuse staining in the rest of the cell. These apoptotic PSF-containing structures were completely inaccessible to the B92 antibody, implying that the antigenic epitope had been blocked. The inaccessibility of PSF in immunofluorescence is not restricted to the B92 mAb only and occurs also with the use of a polyclonal antiserum directed against an antigenic region in the N-terminus, different from the epitopes recognized by B92. These changes are associated with hyperphosphorylation of PSF. However, the B92 antibody efficiently binds to denatured PSF from apoptotic cells, as evidenced by Western blotting of cellular proteins. This indicates that it is not the hyperphosphorylation per se that masks the antigenic sites. Thus, PSF seems to undergo a major conformational change or massive interactions with new partner molecules during apoptosis. The latter possibil-
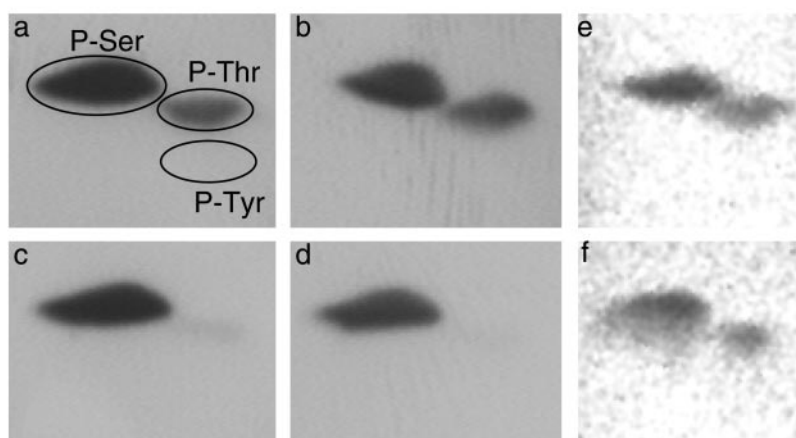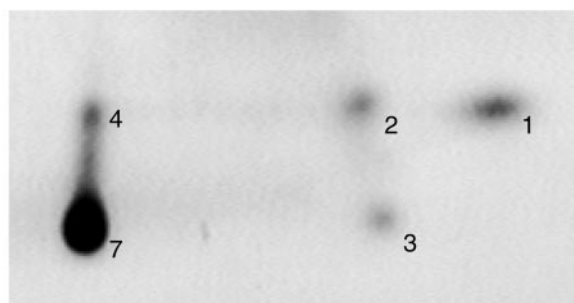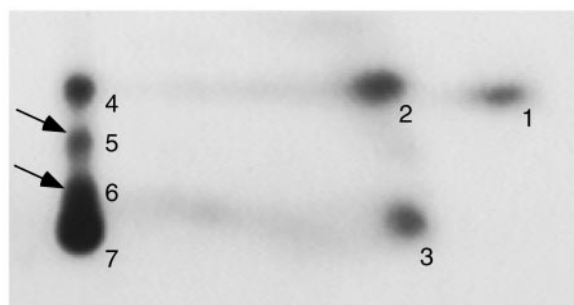
Figure 6. Phosphoamino acid analysis and phosphopeptide mapping of PSF. Phosphoamino acid analysis of PSF immunoprecipitated by B92 antibody from ^{32}P metabolically labeled HeLa and HL-60 cells shows phosphorylation on serine and threonine residues (A): untreated HL-60 cells (a), apoptotic HL-60 cells (b). This analysis of the 68-kDa product of PSF shows only phosphoserine in untreated HL-60 cells (c) and apoptotic HL-60 cells (d). Phosphoamino acid analysis of untreated HeLa cells (e) and mitotic HeLa cells (f). Phosphorylated PSF was immunoprecipitated by B92 antibody, and PSF bands were digested with chymotrypsin and subjected to phosphopeptide mapping. Peptide mixture was loaded on the bottom left corner and the run by electrophoresis in pH 4.72 and then by ascending TLC. Untreated HL-60 cells (B). Apoptotic HL-60 cells (C). Arrowheads, additional phosphorylated peptides.

ity is probably the main cause of this antigenic masking, because during apoptosis PSF dissociated from PTB while associating with U1–70K and SR proteins. It should be considered that during apoptosis PSF assumes a new function and serves as a docking site for several regulatory proteins, which upon recruitment into the PSF apoptotic globules, are removed from the active regulatory machinery.

The PSF protein, both in human and in mouse, is composed of two major regions. The functional C-terminal region contains the two RRMs needed for interactions with RNA (Patton *et al.*, 1993) and for the localization of the protein in speckles (Dye and Patton, 2001). This region is highly conserved between mouse and human. The N-terminal region (first 110 residues) is unusually rich in proline,

glycine, and glutamine residues. In parallel with properties of other proline-rich segments in proteins, this segment is believed to play a role in interactions recruiting other molecules. This region is less conserved between mouse and human in comparison to the C terminus, yet, the major structural elements are evolutionarily conserved. The B92 monoclonal anti-PSF antibody interacts with the N-terminal portion of PSF (Shav-Tal *et al.*, 2000). The antigenic epitope that this antibody recognizes is the tetrapeptide PPXH (Zipori, unpublished observations) that appears three times in the N-terminus of PSF and is conserved between mouse and human, although the amino acids in-between these epitopes vary between the species. Interestingly, in 50% of the cases the unconserved amino acids in the N-terminal region are
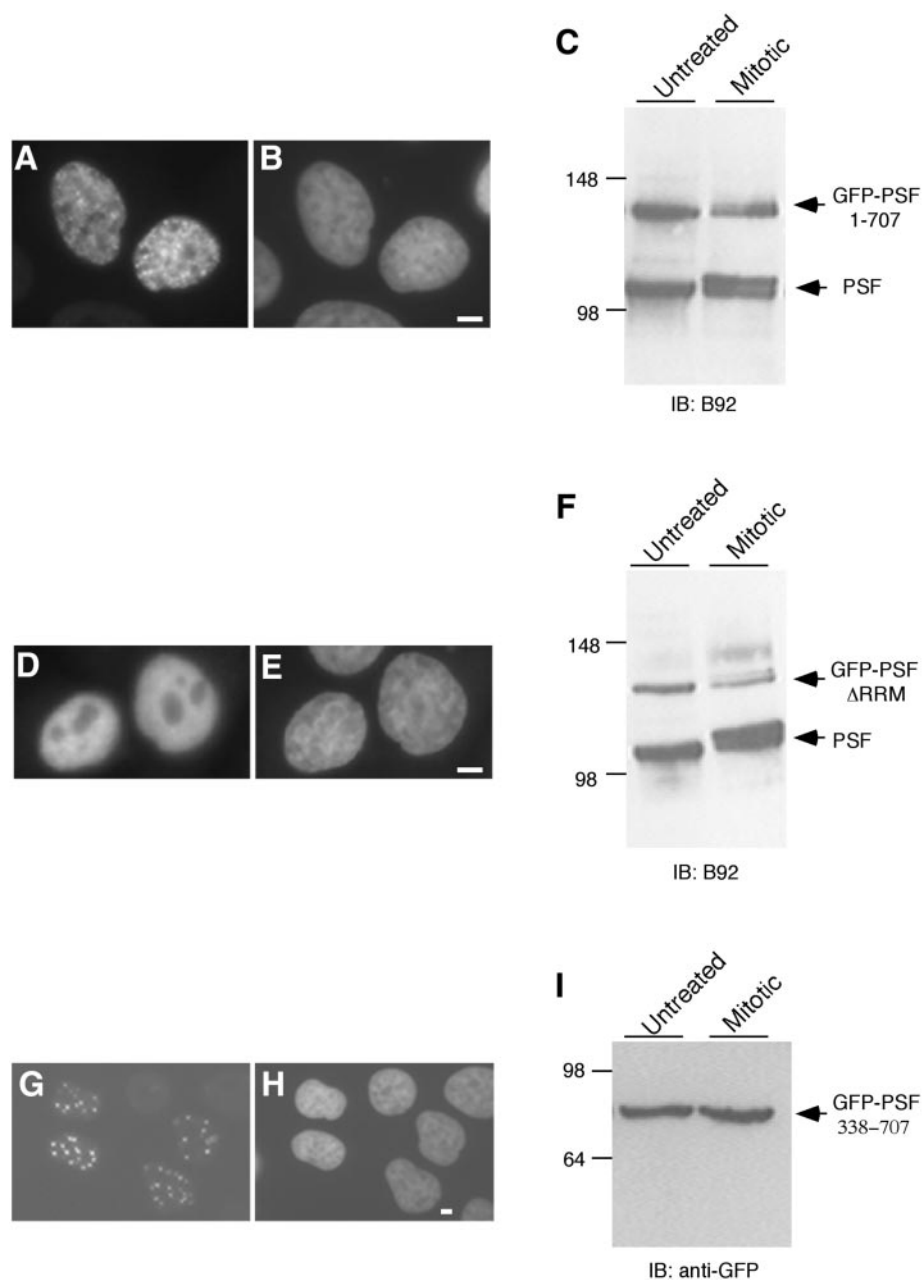
Figure 7. Retarded migration of GFP-PSF constructs. GFP-PSF was transfected into HeLa cells (A), DNA counterstain (B), and endogenic PSF and GFP-PSF were identified in untreated and in metaphase-blocked cells in Western blots with the use of the B92 antibody (C). GFP-PSF ΔRRM2 that does not localize in speckles was transfected into cells (D), DNA (E), and both PSFs were identified as above (F). GFP-PSF aa338–707 was transfected into cells (G), DNA (H), and extracts as above were immunoblotted with an anti-GFP antibody. Bar, 5 μm.

either serine or threonine residues, i.e., putative phosphorylation sites. This may explain some of the differences that we observed between mouse and human PSF. It is likely that protein interactions occurring via this region might mask the antigenic epitope during apoptosis by the phosphorylation of residues in this portion of the protein.

Phosphoamino acid analysis of phosphorylated PSF from mitotic, apoptotic, and untreated cells shows constitutive phos-

phorylation on serine and threonine residues. The same analysis with the 68-kDa degradation product of PSF maps the phosphorylated threonine residues to the C terminus of PSF. Phosphopeptide mapping of phosphorylated PSF shows that there are several additional phosphorylated residues on PSF from apoptotic cells compared with controls possessing an intact nucleus and that the hyperphosphorylation probably occurs on the N-terminus of the protein. This was further

verified by the use of different GFP-PSF constructs: GFP-PSF shows retarded migration in mitotically blocked cells and so does GFP-PSF ΔRRM2, which lacks RRM2, the speckle localization signal. This indicates that the dissociation of PSF from speckles during mitosis probably does not occur because of phosphorylation in this region. GFP-PSF amino acids 338–707 containing only the C terminus did not show any retarded migration, thus implying that hyperphosphorylation of PSF occurs on the N-terminus.

The levels of phosphorylation of PSF as seen by its retarded migration in Western blots are different in mitotic, G1/S-blocked and apoptotic cells. In both mitosis and apoptosis, transcription and pre-mRNA splicing are inactive. This would explain why splicing factors are found in the hyperphosphorylated form, which inactivates their splicing activity. It seems that hyperphosphorylation of PSF occurs at two different levels during mitosis and apoptosis. Untreated cells have intermediate levels of phosphorylation whereas G1/S-blocked cells are hypophosphorylated. Because the higher and lower levels of phosphorylation occur during cell states in which splicing is inhibited, it indicates that both hyper- and hypophosphorylation can be a means for the negative regulation of the PSF protein in pre-mRNA splicing. A similar phenomenon was observed with SR proteins. During early development in nematodes, SR proteins change from a hyperphosphorylated and inactive form to an intermediately phosphorylated and active form (Sanford and Bruzik, 1999). Further dephosphorylation reduces their activity. Similarly, in mammalian cells, both hyper- and hypophosphorylation inhibit the splicing activity of SR proteins (Prasad *et al.*, 1999) and disassemble nuclear speckles (Misteli and Spector, 1996), thus suggesting that intermediate levels of phosphorylation are needed for the functional and structural integrity of these proteins. An alternative interpretation of the differential phosphorylation of PSF, during mitosis and particularly in apoptosis, would be that this protein has functions that are unrelated to splicing. Interestingly, topoisomerase I was shown to specifically phosphorylate SR proteins (Rossi *et al.*, 1996). It therefore may be speculated that the removal of PSF from speckles via hyperphosphorylation is a means for its activation in other cellular functions. Indeed, PSF appears to function in negative regulation of transcription (Urban *et al.*,2000) in the stimulation of topoisomerae I activity (Straub *et al.*, 1998; 2000), in DNA pairing processes that occur during DNA recombination and repair (Akhmedov and Lopez, 2000), and, as shown here, in binding to U1–70K and SR proteins during apoptosis.

The differential phosphorylation may also point to the involvement of different kinases and/or phosphatases during the various cell states. A number of mammalian kinases have been shown to cause disassembly of nuclear speckles and relocalization of splicing factors, and to subsequently affect splicing factor activity. SRPK-1/2, Clk-1/2/3/4, cdc2-kinase, cyclin E-cdk2, topoisomerase I, U1 70-kDa associated kinase, cGMP-dependent kinase, and lamin B-receptor kinase were all shown to phosphorylate SR proteins and other splicing factors in vitro (Woppmann *et al.*, 1993; Gui *et al.*, 1994; Colwill *et al.*, 1996; Nikolakaki *et al.*, 1996; Rossi *et al.*, 1996; Nayler *et al.*, 1997; Duncan *et al.*,1998; Kuroyanagi *et al.*, 1998; Okamoto *et al.*,1998; Seghezzi *et al.*, 1998; Wang *et al.*, 1998; Koizumi *et al.*, 1999; Wang *et al.*, 1999). In addition, some of these kinases were shown to affect the splicing activity of such factors (Mermoud *et al.*, 1994; Cao *et al.*, 1997; Xiao and Manley, 1998; Prasad *et al.*,

1999). How all of these enzymes work in concert in vivo, if at all, is unknown. Cell- and tissue-specific differences in expression of these kinases (Nayler *et al.*, 1997; Kuroyanagi *et al.*, 1998; Wang *et al.*, 1998; Papoutsopoulou *et al.*, 1999) can only provide a partial explanation. A more likely hypothesis is that different kinases are recruited to nuclear speckles during different cell states and phosphorylate splicing factors in a manner specific to their biochemical properties. For example, in the case of the SF2/ASF SR protein, SRPK1 and cdc2 kinase were shown to phosphorylate the protein on different residues (Okamoto *et al.*, 1998). In addition, phosphorylation can differentially modulate protein–protein interactions of several splicing factors (Xiao and Manley, 1998; Yeakley *et al.*, 1999). Thus, although PSF does not contain an RS domain, its cellular localization is controlled in a manner similar to that of SR proteins.

The functional significance of PSF phosphorylation during apoptosis remains unresolved because of the lack of data concerning the fate of splicing factors in programmed cell death. Even though protein phosphorylation is commonly observed in apoptosis (Gjertsen and Doskeland, 1995), the specific roles for this are unknown. It has been suggested that protein phosphorylation targets proteins for cleavage during apoptosis. Such a connection seems to hold in the case of lamin B (Shimizu *et al.*, 1998). Several RNA processing proteins are cleaved by caspases during apoptosis (Wolf and Green, 1999); however, no connection to phosphorylation has been indicated. It was shown that phosphorylated SR proteins associate with the U1-snRNP complex and snoRNPs during apoptosis (Utz *et al.*, 1998; Overzet *et al.*, 2000), and our results show that PSF is another component of this complex during apoptosis. Another study suggests that splicing factors regulate apoptosis, perhaps by regulating caspase activity (Jiang *et al.*, 1998). It is speculated (Prasad *et al.*, 1999) that the phosphorylation of splicing factors at unique residues facilitates the inactivation of these proteins either by rendering them inactive or by irreversibly complexing them with other proteins of the splicing machinery and thus titrating them out of the splicing reaction.

Our data substantiate the concept of the dynamic nucleus that reorganizes its components through a mechanism of phosphorylation, depending on the functional stage of the cell. Our data together with the data accumulated on SR proteins, point to the involvement of several different kinases and phosphatases in the phosphorylation status of nuclear factors. Regulation by phosphorylation is also an end to modulate the activity of the components of the transcription and polyadenylation complexes (Hunter and Karin, 1992; Colgan *et al.*, 1996) and provides a linkage between processes of transcription and splicing (Hirose *et al.*, 1999).

ACKNOWLEDGMENTS

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